

University of Memphis

## University of Memphis Digital Commons

---

Electronic Theses and Dissertations

---

6-2-2010

### **Ferric chloride-induced thrombus formation in murine inferior vena cava requires platelets, platelet receptor GPIb-IX and von Willebrand factor**

Manali Vinay Joglekar

Follow this and additional works at: <https://digitalcommons.memphis.edu/etd>

---

#### **Recommended Citation**

Joglekar, Manali Vinay, "Ferric chloride-induced thrombus formation in murine inferior vena cava requires platelets, platelet receptor GPIb-IX and von Willebrand factor" (2010). *Electronic Theses and Dissertations*. 51.

<https://digitalcommons.memphis.edu/etd/51>

This Thesis is brought to you for free and open access by University of Memphis Digital Commons. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of University of Memphis Digital Commons. For more information, please contact [khggerty@memphis.edu](mailto:khggerty@memphis.edu).

To the University Council:

The thesis committee for Manali V. Joglekar certifies that this is the approved version of the following electronic thesis: “Ferric chloride-induced thrombus formation in the inferior vena cava of mice requires platelets, platelet receptor GPIb-IX and von Willebrand factor”.

---

T. Kent Gartner, PhD  
Major Professor

---

Judith Cole, PhD

---

Carlos E. Lopez-Estrano, PhD

Accepted for the Council:

---

Karen D. Weddle-West, Ph.D.  
Vice Provost for Graduate Programs

FERRIC CHLORIDE-INDUCED THROMBUS FORMATION IN MURINE  
INFERIOR VENA CAVA REQUIRES PLATELETS, PLATELET RECEPTOR GPIB-  
IX AND VON WILLEBRAND FACTOR

by

Manali Vinay Joglekar

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

Major: Biology

The University of Memphis

August 2010

## **Dedication**

This thesis is dedicated to my parents

V.N. Joglekar and M.V. Joglekar

For being a part of all my achievements and having the courage to send me away from  
homeland in pursuit of knowledge



## **Acknowledgements**

I would like to thank all the people without whom this thesis would have been impossible. First I offer my sincerest gratitude to my major advisor, Dr. T. Kent. Gartner. It was an honor to have him as my advisor. He supported me throughout, with his patience, encouragement, knowledge and valuable advices whilst allowing me the freedom to work in my own way. One simply could not wish for a better and jolly supervisor. I would also like to thank my committee members: Dr. Judith Cole and Dr. Carlos Lopez Estrano. Their advice and suggestions were invaluable for my degree completion.

I would like to show my gratitude to Dr. Junling Liu, the former post-doc in our lab without whose guidance and advices the experiments would be incomplete. Special thanks to Mr. Robert Loudon, the then technician in our lab for the help and support.

I would also like to thank Dr. Jerry Ware who provided us different knock-out mice for our studies. I thank Dr. Melinda Fitzgerald for lending us the Blood Perfusion Monitor (BPM) for the blood flow experiments.

Special thanks to Dr. Karyl Buddington and Janet Cooper for helping me in breeding and maintaining mice in the animal facility. I would like to thank Integrated Microscopy staff (Mariallan, Candice and Lou Boykins) for helping me with the histological and SEM studies.

Working was fun with Wendy, Dr. Sridevi Reddy, Gopi, Vamsi always around for help and discussions during coffee breaks. Long hours of studying for tests would not have been possible without Anusha and Lilian.

I would like to acknowledge Donna Haskins and Clara Nunis for their help with all the official documentation.

I would like to thank my parents and my in-laws Mr. M.D. Utturkar and Mrs. A.M. Utturkar for always helping me through the tough times. Thanks to my brother, Vidish with whom I always share good times and have fun. And finally I thank my husband, Gangadhar Utturkar, for constant support, help and love.

## Abstract

M. Joglekar, J. Ware, T. K. Gartner. FeCl<sub>3</sub>-induced thrombus formation in murine inferior vena cava requires platelets, platelet receptor GPIb-IX and von Willebrand factor. XXII Congress July 11-16, 2009. International Society on Thrombosis and Haemostasis. Major Professor: T. Kent Gartner, PhD.

Venous thromboembolism (VTE) is the leading cause of death from cardiovascular disease. Despite the importance of the glycoprotein (GP) Ib-IX/von Willebrand factor (VWF) axis in arterial thrombosis, its requirement in venous, not venule thrombosis has not been tested. We evaluated FeCl<sub>3</sub>-induced thrombus formation in inferior vena cava (IVC) of mice. We first established FeCl<sub>3</sub> injury conditions (20% FeCl<sub>3</sub>, 10 minutes) requiring platelets for IVC occlusion as confirmed by a lack of occlusion in severely thrombocytopenic (less than 23% circulating platelet count) mice (n=7) and stable occlusion in control animals.

Using similar injury conditions, no IVC occlusion was observed using platelet GPIb $\alpha$ -deficient animals (n=6), a model of human Bernard-Soulier syndrome (BSS). Transgenic IL-4R/GPIb $\alpha$  mice were studied to determine if the absence of IVC occlusion in the mouse model of BSS was caused by extracellular GPIb $\alpha$  deficiency or by other platelet abnormalities associated with BSS phenotype. IL-4R/GPIb $\alpha$  mice lack murine GPIb $\alpha$ , but express a transgenic fusion protein compound of an extracellular domains of human IL-4 receptor fused to the transmembrane and cytoplasmic domains of human GPIb $\alpha$  ; an ameliorated form of mouse BSS lacking macrothrombocytopenia. No occlusion was observed in the IVC of IL-4R/GPIb $\alpha$  mice (n=8), just as observed with the murine model of BSS. VWF-deficient mice were also studied and failed to occlude in response to FeCl<sub>3</sub> treatment (n=7).

These data establish experimental conditions for FeCl<sub>3</sub>-induced thrombus formation in the IVC that is platelet, platelet receptor GPIb $\alpha$  and VWF-dependent despite the low venous flow rate in the IVC. Also unlike venules, thrombus formation in IVC's is GPIb-IX dependent, and fibronectin cannot substitute for VWF in IVC's as it can in arterioles.

## Table of Contents

CHAPTER	PAGE
LIST OF FIGURES	x
KEY TO SYMBOLS AND ABBREVIATIONS	xii
1. INTRODUCTION	1
1.1. Deep Vein Thrombosis	2
1.2. Overview of blood coagulation/ haemostasis	3
1.2.1. Tissue factor pathway (extrinsic)	3
1.2.2. Contact activation pathway (intrinsic)	4
1.2.3. Common pathway	4
1.3. Platelets	5
1.3.1. Structure	6
1.3.1a. Plasma membrane	6
1.3.1b. Cytoskeleton	6
1.3.1c. Cytoplasm	7
1.3.2. Platelet receptor GPIb-IX-V	8
1.3.2a. Functions of GPIb-IX-V	9
1.3.3. Ligands of GPIb-IX-V complex	9
1.3.3.1. von Willebrand Factor (major ligand)	9
1.3.3.2. Thrombospondin	10
1.3.3.3. Thrombin	10
1.3.3.4. P- selectin	11
1.3.3.5. $\alpha M\beta 2$	11

1.3.3.6. Non-physiological GPIb $\alpha$ ligands	11
1.3.4. GPIb-IX-V Signaling	12
1.4. von Willebrand Factor	15
1.5. Research approach	16
1.6. Objective	18
 2. MATERIAL AND METHODS	 18
2.1 Animals	18
2.2 Ferric chloride-induced inferior vena cava injury	18
2.2.1. Why FeCl <sub>3</sub> -induced injury model?	19
2.2.2. Why 20% FeCl <sub>3</sub> ?	20
2.2.3. Artery vs. Vein	22
2.3. Laser flow Blood Perfusion Monitor (BPM <sup>2</sup> )	24
2.4. Platelet depletion	24
2.5. Histological studies	25
2.6. Immunohistochemical studies	25
2.7. Statistical analysis	26
2.8. Box plots for the murine blood flow analysis	26
 3. RESULTS	 27
3.1. FeCl <sub>3</sub> treatment of the inferior vena cava causes platelet dependent stable thrombus formation	27
3.2. Platelet receptor GPIb $\alpha$ is required for stable thrombus	

formation in FeCl <sub>3</sub> -induced injury in mouse inferior vena cava	29
3.3. VWF is required for GPIIb/IIIa dependent thrombus formation	
in the mouse inferior vena cava	32
3.4. Histological and Immunohistological studies	33
4. DISCUSSION	36
5. CONCLUSION	40
6. FUTURE SCOPE	41
REFERENCES	42
APPENDIX	
A. Permit for Fig.3	46
B. Permit for Fig.4	47

## List of Figures

Figure	Page
1. Coagulation cascade (normal haemostasis)	5
2. GPIb-IX-V complex	8
3. GPIb-IX-V Signaling and platelet adhesion	13
4. GPIb $\alpha$ signaling resulting in platelet activation and aggregation	14
5. FeCl <sub>3</sub> -induced inferior vena cava injury	19
6A. Changes in blood flow in FeCl <sub>3</sub> treated carotid artery	21
6B. Changes in blood flow in FeCl <sub>3</sub> treated inferior vena cava	21
7A&B Treatment of 10% FeCl <sub>3</sub> for 10 minutes did not occlude the IVC	21
8. Treatment of 20% FeCl <sub>3</sub> to the IVC of WT mice for 10 minutes caused complete occlusion	22
9. Cross section of IVC showing structural difference between an artery (AA) and vein (IVC)	24
10A. 20% FeCl <sub>3</sub> -induced IVC injury to the WT mice treated with Rat IgG (top panel) and anti-GPIb antibody (bottom panel)	29
10B. Box plots of time of occlusion for control mice treated with non-immune IgG and for the mice treated with rat anti-mouse GPIb mabs	29
11A. 20% FeCl <sub>3</sub> -induced injury to the WT mice (top panel) and GPIb KO mice (bottom panel)	31
11B. Box plots of time of occlusion for control mice and GPIb KO mice	31
12A. 20% FeCl <sub>3</sub> -induced injury to the WT mice (top panel) and IL-4R/ GPIb $\alpha$ -tg mice (bottom panel)	32



12B.	Box plots of time of occlusion for control mice and IL-4R/GPIb $\alpha$ -tg mice	32
13A.	20% FeCl <sub>3</sub> -induced injury to the WT mice (top panel) and VWF KO mice (bottom panel)	33
13B.	Box plots of time of occlusion for the control mice and VWF KO mice	33
14A.	5 $\mu$ m thick cross-section of control IVC treated with Diffquick stain (10X)	34
14B.	IVC treated with 20% FeCl <sub>3</sub> for 10 minutes and 5 $\mu$ m thick cross-sections treated with Diffquick stain (100X)	34
15A.	5 $\mu$ m thick control IVC stained with anti-GPIb antibody (10X)	35
15B.	5 $\mu$ m thick cross-section of IVC treated with 20% FeCl <sub>3</sub> for 10 minutes was stained with anti-GPIb antibody (100X)	35

## KEY TO SYMBOLS AND ABBREVIATIONS

DVT- Deep Vein Thrombosis

PE- Pulmonary embolism

VTE- Venous Thromboembolism

VWF- von Willebrand Factor

GPIb-IX-V- Platelet receptor Glycoprotein Ib-IX-V complex

TxA<sub>2</sub>- Thromboxane A<sub>2</sub>

ADP- Adenosine diphosphate

FeCl<sub>3</sub>- Ferric chloride

$\alpha$ Ib $\beta$ 3- Platelet receptor Glycoprotein IIb IIIa

IVC- Inferior vena cava

TF- Tissue factor

TFPI- Tissue factor pathway inhibitor

HMWK- High molecular weight kininogen

ATP- Adenosine tri-phosphate

TSP- Thrombospondin

PSGL-1- P-selectin glycoprotein ligand-1

CHO- Chinese hamster ovary

ECM- Extracellular matrix

SH-2- Src homology domain

SLP-76- SH-2 containing leukocyte protein-76

PI3K- Phosphatidylinositol 3-kinase

BtK- Bruton tyrosine kinase

PLC- $\gamma$ 2- Phospholipase C $\gamma$ 2

PKC- Protein kinase C

IP3- Inositol tri-phosphate

DAG- Diacyl glycerol

cAMP- Cyclic adenosine mono-phosphate

ADAMTS13- A Disintegrin and Metalloprotease with thrombospondin motif 13

KO- Knock- out

BSS- Bernard Soulier syndrome

AA- Abdominal aorta

mab- monoclonal antibody

SPSS- Statistical package for social sciences

CA- Carotid artery

IL-4R/GPIb-tg mice- Interleukin- 4 receptor/GPIb-transgenic mice

+/- - Heterozygote mice

WT- Wild type

## CHAPTER 1: INTRODUCTION

Venous thromboembolism (deep vein thrombosis (DVT) and/or pulmonary embolism, PE) is a leading cause of morbidity and mortality in the United States. VTE causes about 300,000 deaths annually. VTE is a multifactorial disease with both environmental and genetic causal factors [1]. The frequency of VTE increases with increasing age. Therefore the importance of VTE increases as the population ages. The pathogenesis of VTE is poorly understood. This in turn has increased the importance of studying the basis of deep vein thrombosis.

Thrombus formation occurs by different mechanisms in arteries and veins. Thrombus formation in arteries involves platelet interactions with the vessel wall and is shear dependent [2]. Following vascular injury, collagen in the subendothelium is exposed and subsequently plasma von Willebrand factor (VWF) binds to the collagen and is immobilized on the subendothelium. The initial platelet tethering to the injured site is mediated by the interaction of immobilized VWF with platelet receptor GPIb $\alpha$  [3]. Downstream signaling through GPIb results in thromboxane A<sub>2</sub> (TxA<sub>2</sub>) production followed by platelet dense granule secretion which releases ADP. Both TxA<sub>2</sub> and ADP can activate the integrin  $\alpha$ IIB $\beta$ 3 which mediates platelet aggregation and spreading [4]. ADP activates the nearby platelets thereby facilitating the platelet-platelet interactions and increasing the recruitment of more platelets to the site of injury.

Venous circulation operates at low shear (low shear rate, e.g. 50 s<sup>-1</sup> – 100 s<sup>-1</sup>) and it is generally believed that thrombus formation in this bed is less dependent on platelets or shear-dependent interactions with the vessel walls. VTE is thought to arise from changes in the vessel due to stasis [5], inflammation of the vein wall [6], or coagulation

and fibrinolytic abnormalities. In fact, some scientists consider inflammation and/or coagulation to be the initial steps for thrombus formation in veins. Thrombus formation in venules ( shear rate-  $150 \text{ sec}^{-1} - 200 \text{ sec}^{-1}$  ) has been studied and is shown to be VWF dependent but does not absolutely require GPIb [7]. Compared to arteries, shear rates in veins are much lower [8]. Shear rates have a profound effect on the thrombus formation. Although *in vitro* studies have shown that VWF can mediate platelet-vessel wall interactions even at low shear rates [9], the contribution of platelets, GPIb $\alpha$  and VWF in the venous circulation (not venules) has not been characterized. Thus, the objective of this project was to characterize the requirement of platelets, GPIb $\alpha$  and VWF for *in vivo* venous thrombus formation in mice in response to FeCl<sub>3</sub>- induced injury.

To study the contribution of platelet-vessel wall interactions in VTE thrombus formation in murine inferior vena cava (IVC) was studied in response to ferric chloride-induced injury. The results of this study will establish whether or not thrombus formation under conditions of venous shear rate can be dependent on platelets, platelet receptor GPIb-IX-V and VWF.

### **1.1. Deep Vein Thrombosis**

There are two types of veins in the leg; superficial veins and the deep veins. The superficial veins lie just below the skin and the deep veins are located deep within the muscles. In deep vein thrombosis, a blood clot forms in the deep veins of leg. The clot can be formed due to vascular damage or slow or no blood flow (stasis). Sometimes the clot breaks off and travels to the lungs (pulmonary embolism) via venous circulatory system. This process is known as venous thromboembolism (VTE). Risk factors of DVT are age, surgery, trauma, and immobility [1]. People traveling long distances are at risk of

DVT due to immobility in automobiles and aircrafts. The symptoms of DVT are related in part to the limited blood flow to the heart, and include pain in the leg, swelling due to inflammation, warmth and redness.

## **1.2. Overview of Blood Coagulation/ Haemostasis**

Blood coagulation or haemostasis is a complex process that regulates and stops bleeding at the site of vascular injury. Coagulation process depends upon two pathways: Tissue factor pathway (extrinsic) and contact activation (intrinsic) pathway.

Conventionally it is accepted that the initiation of the coagulation pathway is triggered by the extrinsic pathway and the intrinsic pathway amplifies the cascade. These two pathways ultimately lead to fibrin formation (common pathway) that helps to stop bleeding by forming a clot consisting of fibrin and platelets (Fig.1). The two pathways involve activation of an inactive enzyme or zymogen (protease) to its active form which further catalyzes the next reaction in the cascade. The end result is formation of a fibrin mesh which helps to hold platelet plug together.

### **1.2.1. Tissue factor pathway (extrinsic)**

Tissue factor pathway is initiated upon vascular injury/ trauma. Upon the vascular injury, circulating FVII (inactive) comes in contact with the tissue factor (TF) expressed by vascular smooth muscle cells. Under normal conditions, the TF expressed by the vascular smooth muscle cells is masked or separated by the endothelial cells, but after the endothelial cell layer is damaged due to injury, the TF is exposed to the flowing blood and binds to the circulating FVII. Binding of the TF to the FVII forms TF:FVII complex (active). Activated TF:FVII complex activates FIX and FX. Constitutive activation of the TF:FVII complex can prove to be dangerous and thus immediately after the TF:FVII

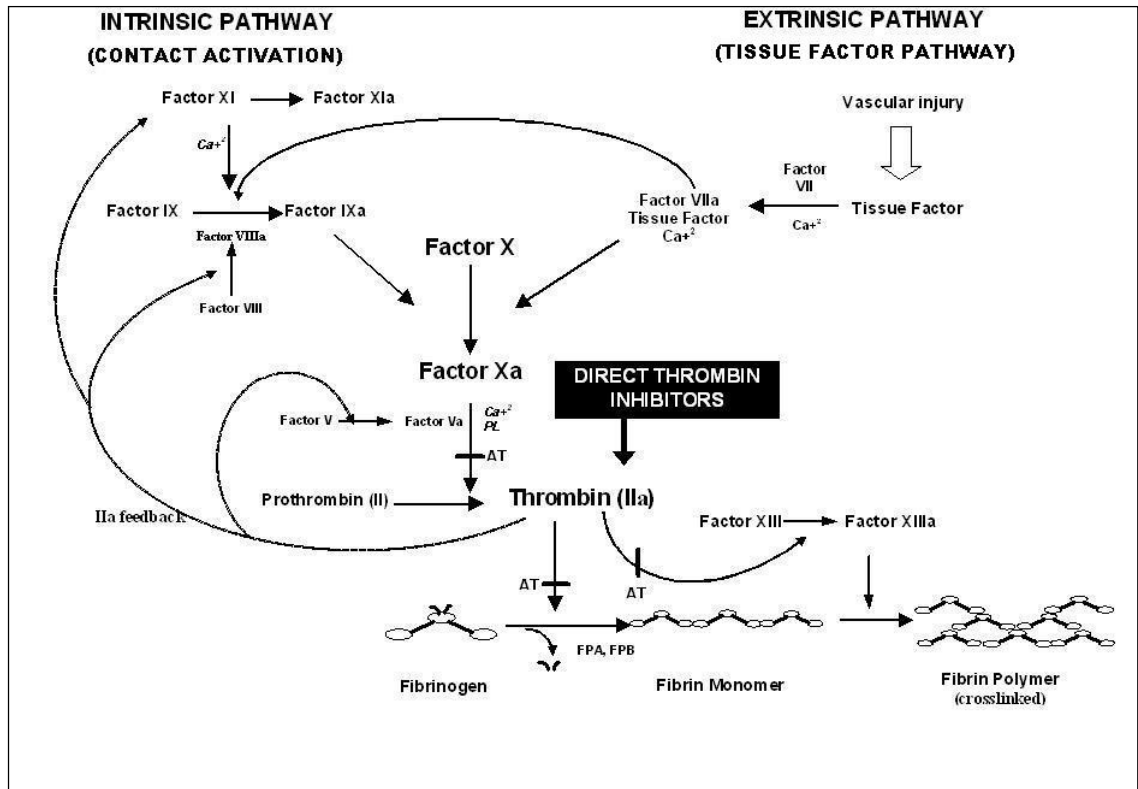
complex activates FX, the TF:FVII complex is inhibited by the tissue factor pathway inhibitor (TFPI). The activated FX (FXa) along with its cofactor FVa forms the prothrombinase complex. Prothrombinase complex further converts prothrombin to thrombin. Further thrombin activates FV and FVIII. FVIIIa is a cofactor for activation of FIX and so the cycle continues.

### **1.2.2. Contact activation (intrinsic) pathway**

This pathway starts with formation of the primary complex on the collagen by a protein called 'high molecular weight kininogen (HMWK), prekallikrein and FXII (Hageman factor). In this pathway, prekallikrein is converted to kallikrein and FXII is converted to its active form (FXIIa). FXIIa activates FXI (FXIa). FXIa in turn activates FIX. FIXa with its cofactor FVIIIa, activates FX to FXa. This explains the amplifying characteristic of the intrinsic pathway. The extrinsic and intrinsic pathways converge at this point into the common pathway.

### **1.2.3. Common pathway**

In this pathway, the thrombin generated by the intrinsic and extrinsic pathway converts fibrinogen to fibrin, which is the building block of the haemostatic plug or a clot.



**Fig.1:** Coagulation cascade (normal haemostasis)

### 1.3. Platelets

Platelets are a critical component of the blood because of their fundamental role in hemostasis and thrombosis. Platelets are anucleate hematopoietic cells. Human platelets are 2.0-5.0  $\mu\text{m}$  in diameter and 0.5  $\mu\text{m}$  in thickness. Normal range of platelet number in blood is  $150-400 \times 10^9$  per liter [10]. Platelets function as cellular “zymogens” dedicated to immediate participation in hemostasis at the sites of vascular injury.



### **1.3.1 Structure**

Platelet structure includes a plasma membrane, the cytoskeleton, and the cytoplasm [10].

#### **1.3.1a Plasma membrane**

The plasma membrane of the platelets consists of a glycocalyx. The exterior surface is a very dynamic structure which serves as the site for the first contact in response to the changes in vascular system. The glycocalyx is mainly composed of the glycoprotein receptors which are necessary for platelet adhesion to vascular surface and to promote platelet aggregation and interaction with the other blood components. The plasma membrane is made up of the lipid bilayer.

#### **1.3.1b Cytoskeleton**

The cytoskeleton consists of microtubules and microfilaments. The microtubules are present in bundles ranging from 3 to 24 and are present very close to the plasma membrane. The major protein constituent of microtubules is tubulin. Microfilaments are made up of globular protein called actin. These filaments play a vital role in platelet physiology. Microfilaments are present near both the plasma membrane and are the major constituent of the cytoplasmic cytoskeleton. The microfilaments associated with the plasma membrane serve as matrix on which the organelles are suspended. The cytoskeleton helps the secretion of the contents from the  $\alpha$ -granules and dense bodies to the exterior after the platelet activation.

### 1.3.1c Cytoplasm

Platelet cytoplasm contains  $\alpha$ -granules, dense bodies, lysosomes, mitochondria.

**$\alpha$ -granules-** There are 40 to 80  $\alpha$ -granules per platelet. They are round to oval shaped, surrounded by a membrane and are about 200 to 500 nm in diameter. The granules contain more than two dozen different proteins which play a role in hemostasis, thrombosis and wound healing such as adhesive proteins, chemokines, cytokines, coagulation factors, angiogenic regulatory proteins and fibrinolytic proteins etc.

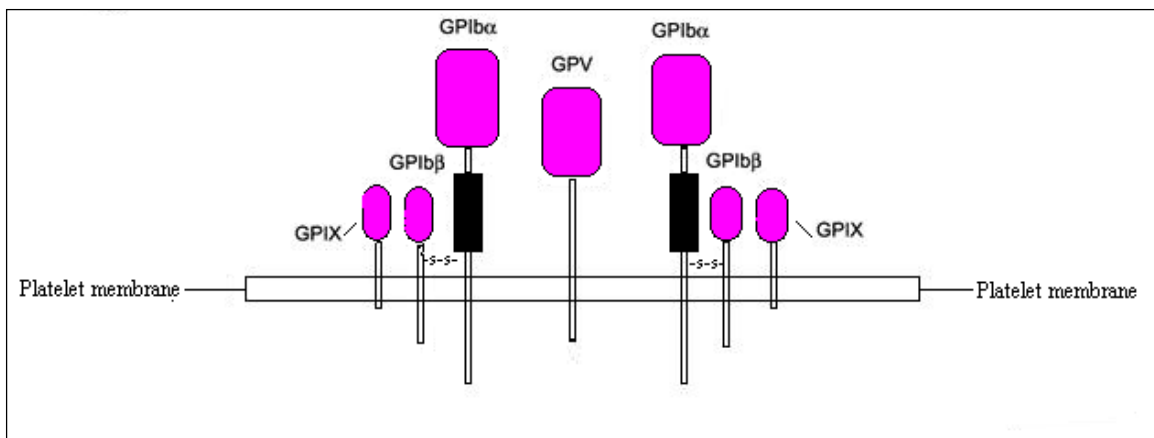
**Dense bodies-** Dense bodies are smaller and less numerous than the  $\alpha$ -granules. Dense bodies are spherical and covered with an enclosing membrane. Some dense bodies can have whiplike extensions. On an average, there are 4-8 dense bodies in a platelet. Dense bodies are rich in adenine nucleotides such as ATP and ADP, serotonin, calcium, magnesium and pyrophosphates.

**Mitochondria-** There are very few mitochondria present in a platelet. These are very simple structurally and are the sites of oxidation in the platelets.

**Lysosomes-** Human platelets have few lysosomes (minimum 0 to 1, maximum 3). They have 13 acid hydrolases and cathepsin D and E and CD36 (a lysosomal membrane protein). The function of platelet lysosomes is not clear but they release their contents after platelet activation. Platelet lysosomes do not engulf bacteria as do phagocytes so they are considered as vestigial organelles with no clear function in platelets.

### 1.3. 2. Platelet Receptor Glycoprotein IB-IX-V

The glycoprotein Ib-IX-V complex is an important platelet membrane adhesion receptor that participates in both haemostasis and thrombosis. As shown in figure 2, the GPIb-IX complex is made up of the following four different gene products: GPIb $\alpha$  (~ 135 kDa), which is linked to GPIb $\beta$  (~25 kDa) by disulfide bonds, GPIX (~22 kDa) and GPV (~82 kDa). The GPIX and GPV components are attached to the GPIb by non-covalent interactions. These four subunits make the intact GPIb-IX-V complex as shown in figure 2. This complex is expressed on the platelet surface in the ratio of 2:2:2:1 for GPIb $\alpha$ : GPIb $\beta$ : GPIX: GPV. About 25,000 GPIb-IX-V complexes are constitutively expressed on the platelet surface [3]. In humans, GPIb, IX, and V are type I membrane spanning subunit having a N-terminal ligand binding domain, a transmembrane region and cytoplasmic tail as shown in figure 2. The N-terminal domain of the GPIb $\alpha$  contains the VWF binding domain.



**Fig. 2:** Glycoprotein Ib-IX-V complex  
(Modified from: [journals.prous.com/journals/servlet/xmlxsl/pk...](http://journals.prous.com/journals/servlet/xmlxsl/pk...) )

### **1.3.2a Functions of GPIb-IX-V**

The main functions of the platelet receptor GPIb-IX-V complex are as follows:

1. Regulation of platelet adhesion to the sub-endothelial layer and leukocytes.
2. Assemble/procoagulant activity on activated platelets.
3. Initiate outside-in signaling that activates platelets and platelet  $\alpha\text{IIb}\beta\text{III}$  resulting in platelet aggregate formation at the site of platelet adhesion to the damaged endothelium. (Topographical interactions) [11].

### **1.3.3. Ligands of GPIb-IX-V complex**

VWF is the major ligand for the receptor GPIb. Amongst the other ligands includes thrombospondin (TSP), thrombin and P-selectin. There are a variety of non-physiological ligands for this receptor such as snake venoms toxins and bacteria [12].

#### **1.3.3.1 von Willebrand factor (major ligand)**

Interaction of soluble (that which circulates in the blood) VWF with its receptor GPIb is shear dependent. At all arterial shear rates, the GPIb $\alpha$ -VWF interaction is required for platelet adhesion to the damaged vascular surface [13]. The interaction of GPIb $\alpha$  and VWF has been studied by crystallography. VWF binds to the N-terminal ligand binding region of GPIb $\alpha$  by its A1 domain [14]. GPIb $\alpha$  residues have a direct contact with the VWF A1 domain and are clustered toward the N-terminal and C-terminal ends of the ligand binding region [12]. VWF is the major ligand of glycoprotein receptor Ib $\alpha$ . Binding of VWF to its receptor GPIb $\alpha$  is essential, as it elicits the initial step for the platelets to become stationary on the injured vascular wall and thus for the thrombus growth. Binding of VWF to GPIb $\alpha$  also triggers signaling events that lead to

platelet activation, as well as cytoskeletal changes that regulate platelet spreading, shape change, secretion and aggregation. Under normal circumstances, soluble VWF does not bind stably to GPIb $\alpha$  on platelets suspension [12]. Normal interaction of GPIb $\alpha$  with VWF requires a trigger which can be pathological shear stress, immobilization of VWF on collagen or other subendothelial components. After VWF-mediated adhesion of platelets to the damaged endothelium, secreted VWF can bind to active  $\alpha$ IIb $\beta$ 3 in the growing aggregate thereby facilitating hemostasis or thrombus formation [12].

#### **1.3.3.2 Thrombospondin**

Thrombospondin-1(TSP) is a recently identified ligand for GPIb-IX-V. TSP binds GPIb-IX-V at high shear rate and is able to support platelet adhesion in absence of VWF [15].

#### **1.3.3.3 Thrombin**

Thrombin binds to the  $\alpha$ -subunit of GPIb at the Tyr276 residue, an interaction important for normal physiological hemostasis and thrombosis. *In vivo* thrombus formation was studied for both laser induced injury mouse model and FeCl<sub>3</sub> induced injury model using the transgenic mice expressing the normal human GPIb $\alpha$  (hTg<sup>WT</sup>) subunit and a mutant human GPIb $\alpha$  containing a Phe substitution for the Tyr276 (hTg<sup>Y276F</sup>). Delayed thrombus formation in the mutant mice (hTg<sup>Y276F</sup>) in the FeCl<sub>3</sub> induced injury and less stable thrombus in laser induced injury demonstrates the physiological importance of this interaction [16].

#### **1.3.3.4 P-selectin**

P-selectin is a type I transmembrane protein which is expressed on the activated endothelial cells. It is present in both the endothelial cells (Weibel-Palade bodies) and in the  $\alpha$ -granules of platelets. P-selectin recruits leukocytes (through its P-selectin glycoprotein ligand-1 [PSGL-1] receptor) to the activated endothelial cells in an inflammatory response. Studies have shown that, like leukocytes, P-selectin can also bind to the platelet GPIb $\alpha$ . Adhesion studies carried out in the CHO (Chinese Hamster Ovary) cells expressing P-selectin show that these cells adhere to the immobilized GPIb $\alpha$  (glycocalicin) in a P-selectin dependent manner [17]. Thus it has been shown that the platelet GPIb $\alpha$  and P-selectin interactions may have a potential role in pathological conditions.

#### **1.3.3.5 $\alpha$ M $\beta$ 2**

$\alpha$ M $\beta$ 2 is a leukocyte integrin and it is also known to be a ligand for platelet receptor GPIb $\alpha$ . The  $\alpha$ M $\beta$ 2-GPIb $\alpha$  interaction is required for adhesion of leukocytes to the growing thrombus at the injured vascular bed [18]. The  $\alpha$ M $\beta$ 2-GPIb $\alpha$  interaction also facilitates physiological clearance of cold stored (<15° C) platelets. This clearing is due to the cold induced clustering of GPIb $\alpha$  which leads to clearance of platelets by phagocytic liver macrophages or Kupffer cells [19].

#### **1.3.3.6 Non-physiological GPIb $\alpha$ ligands**

Bacteria can bind to the platelet specific receptor GPIb $\alpha$ . Binding of bacteria to the receptor can be of physiological significance and can increase the risk of thrombosis. GPIb $\alpha$  has high amount of sialomucin. Some bacteria recognize the sialic acid by their cell wall proteins, e.g. *Streptococcus gordonii*. Some bacteria such as *Staphylococcus*

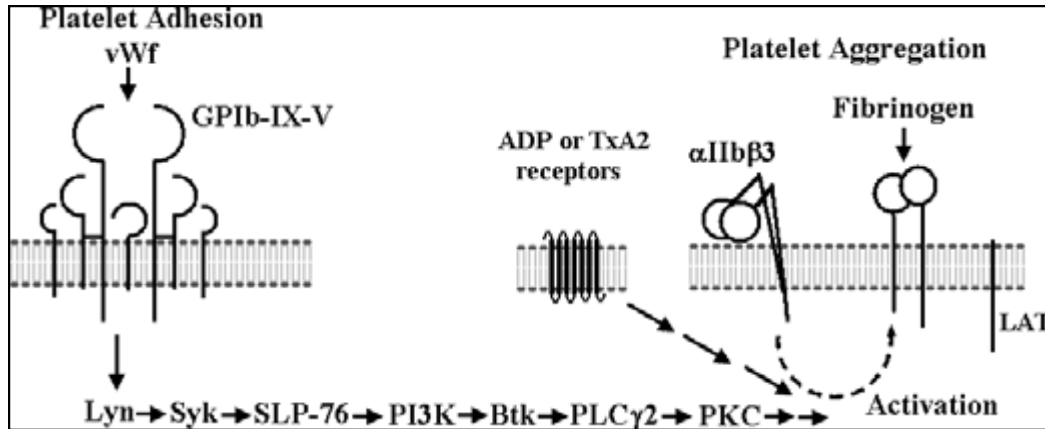
*aureus*, bind the GPIIb $\alpha$  receptor by forming bridging interactions between GPIIb $\alpha$  and VWF. Other bacteria bind directly to the N-terminal domain of GPIIb $\alpha$  [12].

Snake venoms are also one of the non-physiological ligands of GPIIb $\alpha$ . Snake venoms are complex mixtures of active proteins and peptides. Based on their protein/peptide sequence, they are classified as serine proteases, metalloproteinases, C-type lectins, disintegrins or phospholipases. They take part in hemostasis by either activating or inhibiting platelet functions, coagulant factor functions or disrupting the endothelial cells (e.g. metalloproteinases in the Crotalidae or Viperidae degrades the blood vessel extracellular matrix [ECM] thus affecting the haemostasis or Rhodostomin [a disintegrin] inhibits steps in angiogenesis and also can induce endothelial cell apoptosis.)The snake venoms activate platelets by activating protease receptors, or by binding or degrading VWF and platelet receptors or by altering the TxA<sub>2</sub> production [20].

#### **1.3.4. GPIIb-IX-V Signaling**

Thrombus formation in vessels with high shear rates such as in arteries is dependent on the platelet adhesion receptor GPIIb-IX-V [2, 3 and 4]. Upon vascular injury, the collagen in the subendothelium is exposed and circulating VWF binds to the collagen. VWF-GPIIb initial interaction triggers GPIIb dependent signaling (Fig.3) which results in TxA<sub>2</sub> production. This signaling is initiated by Lyn (a tyrosine kinase), Syk (a cytoplasmic tyrosine kinase), SLP-76 (Src homology 2 (SH2) domain containing leukocyte protein76 (SLP-76), Phosphatidylinositol 3-kinase (PI3K), Bruton tyrosine kinase (Btk), Phospholipase C $\gamma$ 2 (PLC $\gamma$ 2) and protein kinase C (PKC) which ultimately

activates receptor integrin  $\alpha\text{IIb}\beta 3$  which leads to platelet aggregation and secretion (Fig.3).

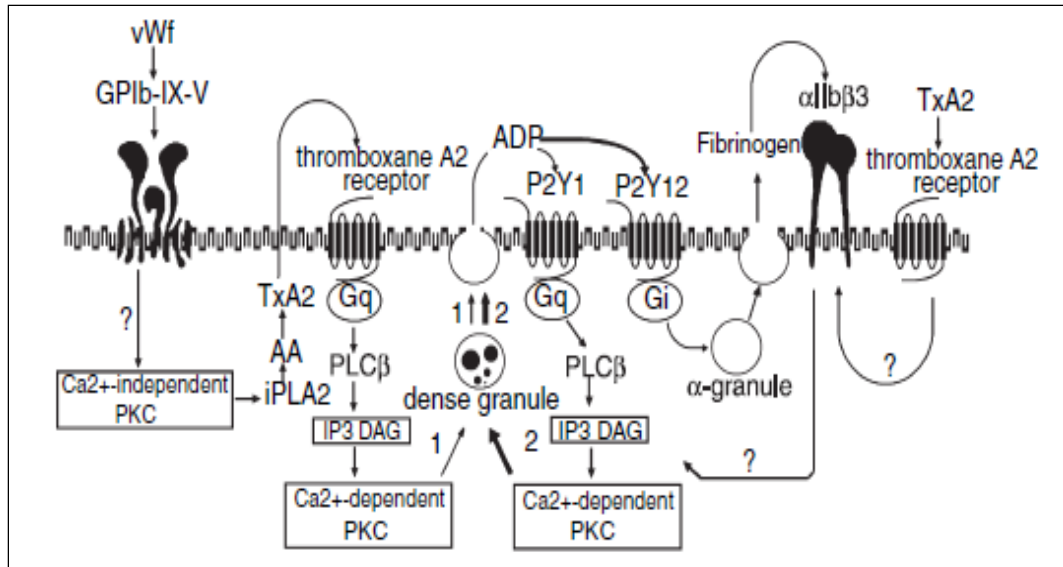


**Fig.3:** GPIb-IX-V Signaling and platelet adhesion

**Source:** J. Liu, M. Joglekar, J. Ware, M. E. C. Fitzgerald, C.A. Lowell, M. C. Berndt and T. K. Gartner. Evaluation of physiological significance of botrocetin/von Willebrand factor *in vitro* signaling. 2008; **6**: 1915-1922.

Thus the VWF-GPIb initial interaction induces a cascade of events including protein phosphorylation, calcium elevation, phospholipase activation, TxA2 production and ADP secretion. These events lead to platelet aggregation that can be a part of normal hemostasis or thrombus growth at the site of injury [4].





**Fig.4:** GPIIb $\alpha$  signaling resulting in platelet activation and aggregation.

**Source:** J. Liu, T.I. Pestina, M.C. Berndt, S. A. Steward, C. W. Jackson and T. K. Gartner. The roles of ADP and TxA2 in botrocetin/VWF-induced aggregation of washed platelets. *J Thromb Haemost.* 2004; **2**: 2213- 2222.

Fig.4, shows in detail the events induced by GPIIb $\alpha$ - initiated signaling.

Activation of GPIIb $\alpha$  leads to thromboxane A2 (TxA2) production and secretion. TxA2 then binds to its G-protein coupled receptor which functions through G $\alpha_q$  activation. G $\alpha_q$  recruits PLC $\beta$  which in turn produces inositol- trisphosphate (IP3) and diacyl glycerol (DAG). IP3 and DAG mobilize the intraplatelet Ca<sup>2+</sup> which in turn activates protein kinase C (PKC). PKC triggers dense granule secretion. ADP is released along with the other contents of dense granules. ADP binds to G- protein coupled P2Y1 and P2Y12 receptors which function through G $\alpha_q$  and G $\alpha_i$  respectively. The signaling through G $\alpha_q$  further amplifies the dense granule secretion and the signaling through G $\alpha_i$  promotes  $\alpha$ -

granule secretion by inhibiting cAMP production. Fibrinogen present in the  $\alpha$ -granules is released and binds to its receptor,  $\alpha$ Ib $\beta$ 3. These events lead to platelet aggregation [4].

#### **1.4. von Willebrand Factor (VWF)**

VWF is a large multimeric adhesive glycoprotein. It is present in plasma, endothelial cells (Weibel Palade bodies), megakaryotes, platelet  $\alpha$ -granules and in connective tissue. It is secreted through constitutive and regulatory (secretory) pathways. It is constitutively secreted from the Weibel Palade bodies in the endothelial cells. It has been shown that the VWF present in plasma is structurally different from that present in the platelet  $\alpha$ -granules. The platelet VWF is larger than the one present in plasma. This structural difference can play a specific role in normal haemostasis [21]. A mature VWF molecule is about residues or 275 kDa. It consists of linear sequence of modular domains as designated: D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. The VWF in Weibel Palade bodies in endothelial cells is ultra-large VWF and is hyperadhesive. The ultra-large VWF is processed to smaller, less adhesive forms of VWF by proteolysis by (A Disintegrin and Metalloprotease with Thrombospondin motif) called ADAMTS13 [12]. VWF plays a role in normal haemostasis as it promotes platelet adhesion to the site of vascular injury and platelet-platelet cohesion for the thrombus growth. It is also a carrier for coagulation Factor VIII in plasma and protects it from proteolysis thus facilitating thrombin generation. The smaller VWF (multimers) are secreted in the circulation from large complexes of protein at the time of or after secretion from endothelial cells [12]. VWF acts as a major ligand for the platelet specific receptors GPIb-IX-V and  $\alpha$ Ib $\beta$ III as shown in Fig.3. VWF/GPIb $\alpha$  interaction mediates platelet tethering to the subendothelium in

arteries, arterioles and venules [22], but the requirement for VWF and GPIb $\alpha$  in stable thrombus formation in veins has not been reported.

### **1.5. Research Approach**

A mouse model of ferric chloride-induced injury to the inferior vena cava (IVC) was used to study thrombus formation under venous shear rates. The requirements of platelets, the platelet specific receptor GPIb $\alpha$  and its ligand VWF were characterized using 'knock-out' mice. KO mice are genetically engineered mice in which a particular structural gene for a specific protein is subjected to a major structural alteration that results in permanent loss of the normal gene product. KO mice lacking the specific protein are used to evaluate the importance of that protein in signaling.

Lack of thrombus formation in the knockout mice in response to FeCl<sub>3</sub>-induced injury shows the requirement for the missing function for a stable thrombus formation.

### **1.6. Objective**

The immediate objective of this project is to evaluate the requirements for platelets, the platelet specific glycoprotein Ib $\alpha$  receptor and VWF in FeCl<sub>3</sub>-induced stable thrombus formation in the mouse inferior vena cava.

This objective will be accomplished by completing following specific aims:

1. Evaluate ferric chloride-induced thrombus formation in the IVC in mice with thrombocytopenia (22 % of the normal platelet count) induced by treatment with a mixture of rat anti-mouse GPIb monoclonal antibodies that elicit a rapid depletion of host animal's platelets. Assuming that platelets are required for stable thrombus formation in IVC, Aim 2 will be completed, if not, Aim 4 will be completed in absence of completion of Aim 2 and 3.

2. Evaluate ferric chloride-induced stable thrombus formation in GPIb KO mice.

The platelets produced in these mice lack GPIb, resulting in a condition known in humans as Bernard Soulier syndrome (BSS). The mice suffering from BSS produce abnormal platelets as a consequence of lacking GPIb. Assuming that GPIb is required for ferric chloride-induced stable thrombus formation in the IVC, Aim 3 will be completed.

3. Evaluate  $\text{FeCl}_3$ -induced stable thrombus formation in transgenic mice which lack murine GPIb, but express a chimeric form of human GPIb. The chimeric protein is designed to eliminate the extracellular domain of GPIb, without disrupting the normal non-stimulated function of the cytoplasmic domain of GPIb. Expression of transgenic GPIb mice ameliorates the abnormalities associated with the GPIb deficiency (BSS).
4. Evaluate  $\text{FeCl}_3$ -induced stable thrombus formation in VWF KO mice. These mice very closely mimic the severe or type 3 human von Willebrand disease. Type 3 von Willebrand disease is characterized by absence of VWF in plasma, platelets, endothelial cells and subendothelium. Therefore these mice exhibit defects in hemostasis and show highly prolonged bleeding times.

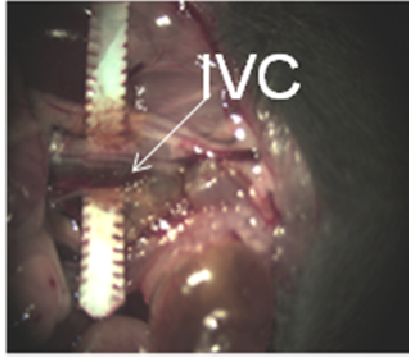
## **CHAPTER 2: MATERIAL AND METHODS**

### **2.1. Animals**

Mice deficient in the platelet receptor glycoprotein Ib $\alpha$ , and the IL-4R/GPIb transgenic mice were obtained from Dr. Jerry Ware's laboratory at the University of Arkansas for Medical Sciences (Little Rock, AR) [23], [24]. The IL-4R/GPIb $\alpha$ -tg mice lack murine GPIb $\alpha$ . They express the extracellular domain of human IL-4 receptor fused with the transmembrane and cytoplasmic domains of human GPIb $\alpha$ . The von Willebrand factor KO mice [25] were bred in our animal facility (University of Memphis) from breeding stock obtained from Jackson Laboratory, Maine. Heterozygotes of these KO strain have been backcrossed with C57Bl/6 mice for at least eight generations.

### **2.2. Ferric chloride-induced inferior vena cava injury**

Mice were anesthetized using isofluorane (Baxter International Inc, Deerfield IL). The intact IVC was dissected free from surrounding tissue and placed on a paper strip covered with adhesive tape (13×3 mm), for support (Fig.5). Injury was induced by topical application of a longitudinally folded strip of Whatman No. 1 filter paper (14×3mm) saturated with 20% FeCl<sub>3</sub> [26, 27] (J.T Baker Co; NJ) for 10 minutes at room temperature. Then the tissue was rinsed with physiological saline to remove FeCl<sub>3</sub>. Blood flow was monitored using a laser Doppler system with a pencil probe. Monitoring of blood flow lasted for 45 minutes starting at the time of the application of FeCl<sub>3</sub>. Occlusion was defined as a decrease of blood flow of 75% or more for at least 3 minutes [28].



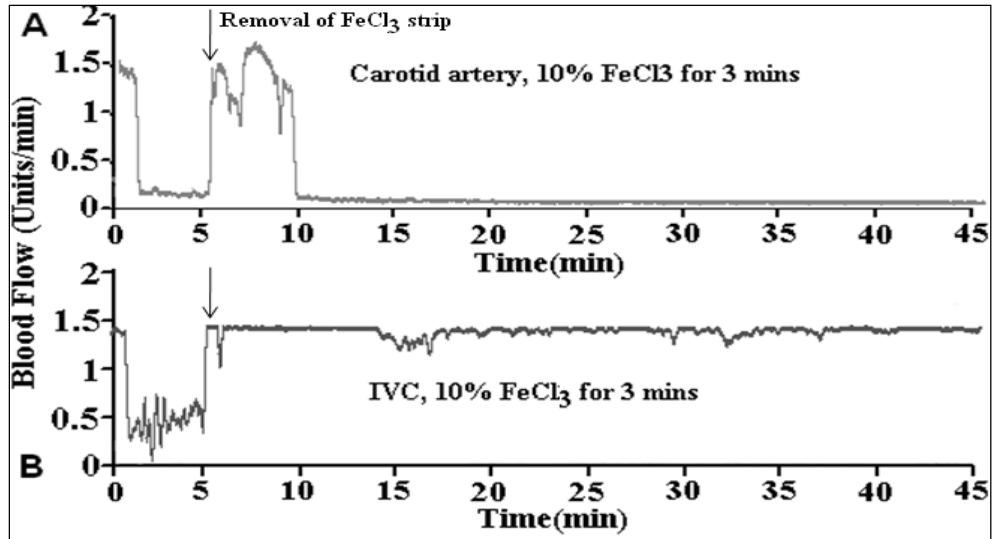
**Fig.5:** Ferric chloride-induced inferior vena cava injury. (The arrow indicates the inferior vena cava separated on the paper strip covered with adhesive tape).

### **2.2.1 Why ferric chloride induced injury model**

Ferric chloride ( $\text{FeCl}_3$ ) is often used to induce injury in mice and other animals. The murine model for thrombus formation in response to ferric chloride induced injury is well characterized [29].  $\text{FeCl}_3$  induces injury to the vessel by oxidizing the vessel and rupturing the endothelial layer and thus exposing collagen [27]. Furthermore, thrombus formation in response to  $\text{FeCl}_3$  in the carotid artery is GPIIb $\alpha$ -dependent [30, 31]. Lack of stable thrombus formation was observed in mice lacking the extracellular domain of the platelet receptor GPIIb $\alpha$  (which is the major ligand binding domain) after treating the carotid artery with 10%  $\text{FeCl}_3$  [28]. These data demonstrate that stable thrombus formation in response to  $\text{FeCl}_3$  induced injury is GPIIb $\alpha$  dependent in the carotid arteries. Therefore  $\text{FeCl}_3$ -induced injury model was an appropriate model to characterize the requirement for platelets, the platelet specific receptor GPIIb $\alpha$  and VWF in thrombus formation under venous shear rates.

### **2.2.2 Why 20% ferric chloride**

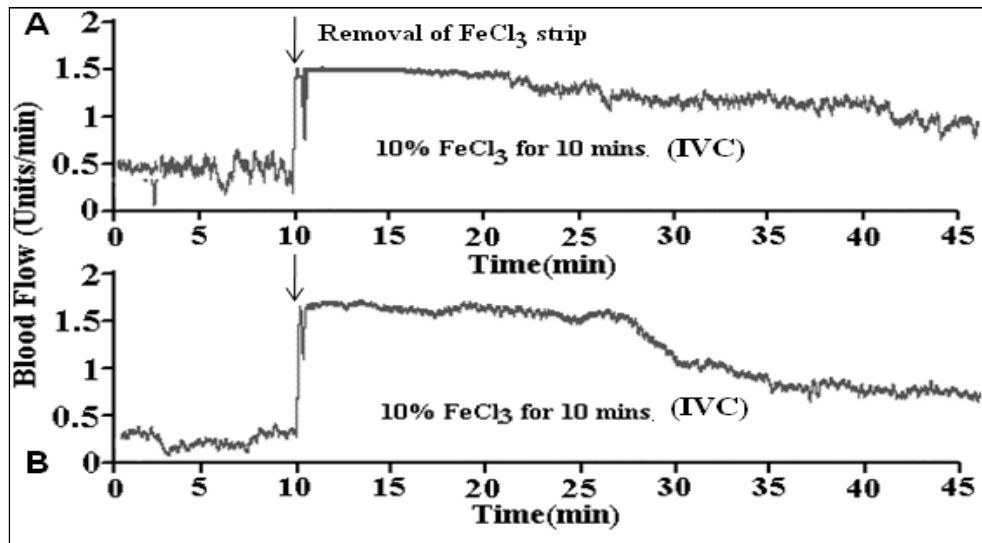
Stable occlusion in the murine carotid artery occurs reproducibly in response to treatment with 10%  $\text{FeCl}_3$  for 3 minutes, with the mean time of occlusion of ~ 5.0 minutes [28] (Fig.6A). Therefore, initially exposure to 10% ferric chloride for 3 minutes was used for the IVC studies. This treatment procedure did not elicit stable occlusive thrombi in the IVC. None of the mice treated with 10% ferric chloride solution for 3 minutes showed occlusion (Fig.6B). Next, the time for exposure was increased from 3 minutes to 10 minutes keeping the concentration of  $\text{FeCl}_3$  the same (10%). The increase in the exposure time did not result in stable thrombus formation (Fig.7A, B). Therefore, the concentration of ferric chloride was increased to 20% keeping the time for exposure as 10 minutes. This concentration of ferric chloride resulted in complete and stable occlusion of the IVC (Fig.8). The results were reproducible.



**Fig.6: Changes in blood flow in FeCl<sub>3</sub> treated carotid artery (A) and inferior vena cava (B).**

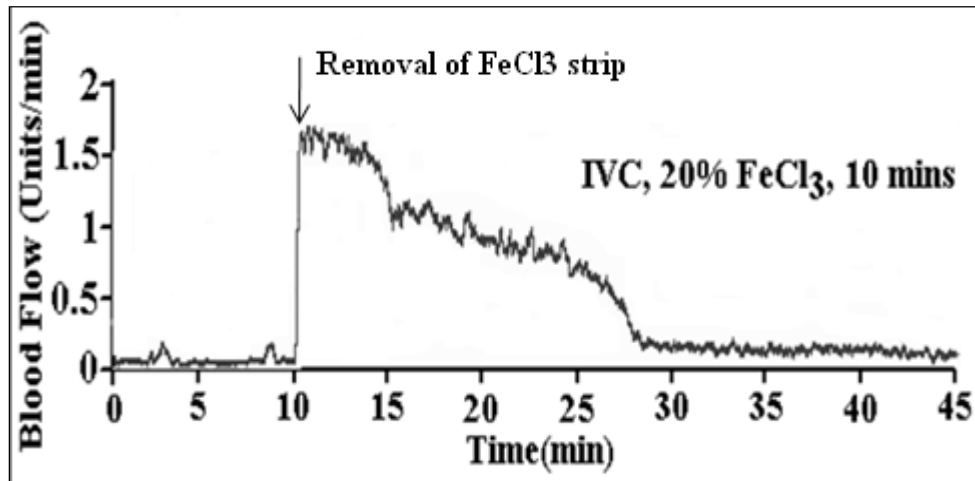
(A): A 3 minutes treatment with 10% FeCl<sub>3</sub> causes stable thrombus formation in a WT mouse within 5 minutes.

(B): 10% FeCl<sub>3</sub> treatment for 3 minutes does not occlude the IVC.



**Fig.7A&B: Treatment of 10% FeCl<sub>3</sub> for 10 minutes did not occlude the IVC.**





**Fig. 8:** Treatment of 20% FeCl<sub>3</sub> to the IVC of WT mice for 10 minutes caused complete occlusion.

Therefore treatment of the IVC with 20% ferric chloride treatment for 10 minutes was used for the following study. Our studies showed that 10% ferric chloride was sufficient to elicit the formation of stable thrombi in arteries, but was not sufficient to elicit stable thrombus formation in IVC. The reason(s) for this difference in response is/are not understood.

### 2.2.3 Artery vs. Vein

A comparison of the structural features of arteries and veins is presented because, it is considered that mechanism of thrombus formation in veins is essentially different from that in arteries. Also there is a physiological difference in between these two types of vessels (Fig.9).

The walls of arteries and veins are made up of three layers which surround the lumen:

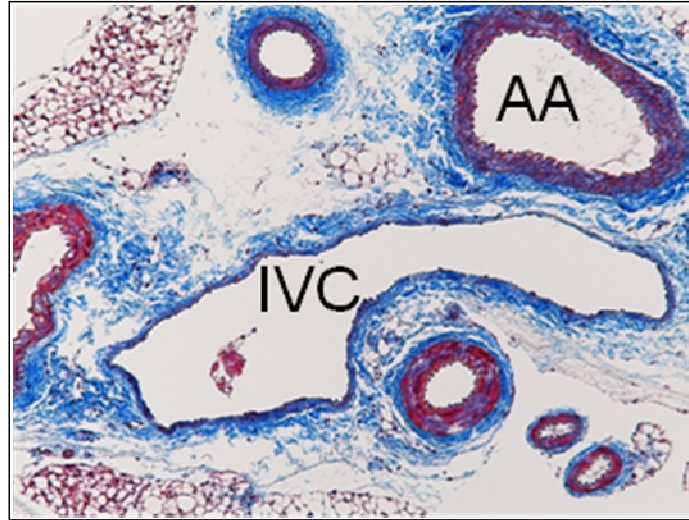
- a. Tunica interna/intima- This is the innermost layer which is made up of endothelial cells.

- b. Tunica media- This is the middle layer made up of circularly arranged smooth muscle cells and sheets of elastin. This layer is generally thicker in arteries than in veins.
- c. Tunica externa- This is the external layer composed of loosely meshed collagen fibres. These fibers protect the vessel and help in anchoring the vessels to the surrounding structures.

These three layers of the vessels differ in the diameter and the thickness according to the type of blood vessel (arteries, arterioles, veins, venules). The shear rates in arteries and veins are different (Table 1). Veins have thinner elastic fiber and smooth muscle layers than arteries and have thin smooth muscle layer [30].

**Table 1** Difference in arteries and veins [32]

<b>Artery</b> <b>(Resistance Vessels)</b>	<b>Vein</b> <b>(Capacitance vessels)</b>
Thick smooth muscle layer ( tunica media)	Thin smooth muscle layer (tunica media)
Small diameter/lumen Elastic artery- 1.5 cm Muscular artery- 6.0 mm	Large lumen/diameter Vein- 5.0 mm
High shear rates (~ 1500 sec <sup>-1</sup> )	Low shear rates (50-100 sec <sup>-1</sup> )
Muscular and thick walls; resistant to stretching	Veins are much flexible in nature



**Fig.9:** Cross-section of IVC showing structural differences between an artery (AA- abdominal aorta) and vein (IVC- inferior vena cava). The section was stained with Masson's trichrome stain. (Magnification: 10 X)

### **2.3. Laser flow Blood Perfusion Monitor (BMP<sup>2</sup>)**

The laser flow blood perfusion monitor uses laser doppler technology. A low power beam of laser light is delivered to the tissue via a fiber optic pencil probe. The optic fibers in the pencil probe collect the light signals carrying information. The probe is connected to the blood perfusion monitor which is in turn is connected to the computer. Graphical representation of the blood flow is obtained from the computer. The final results are local blood flow rates.

### **2.4. Platelet depletion**

An Fc receptor-independent decrease in mouse platelets was elicited by the injection of a mixture of rat anti-mouse GPIb monoclonal antibodies (mabs) into the tail veins of mice [33]. Each isofluorane anesthetized mouse was dissected to expose the IVC prior to treatment (to prevent excess bleeding) with the anti-mouse GPIb mabs (1µg/g of the anti-GPIb $\alpha$  antibody; Emfret Analytics, Germany). Monoclonal antibody

(mab) was injected into a tail vein. Less than 23% of the platelets remained 15 minutes after injection of the antibodies (data not shown).  $\text{FeCl}_3$  treatment of the exposed IVC was initiated 15 minutes after injection of the anti-GPIb antibodies. As a negative control for platelet depletion, 1  $\mu\text{g/g}$  of non-immune rat IgG (Emfret Analytics, Germany) was injected in the tail vein after exposing the IVC.

## **2.5. Histological studies**

For the histological studies, the tissue (IVC) was fixed in 16 % paraformaldehyde for 24 hours at 4°C. The tissue was further processed by dehydrating the samples in increasing concentrations of ethanol (70% for 30 min, 95% 2×30 min, absolute ethanol 3×30 min). The tissue was then cleared with xylene 3×30 min and infiltrated with paraffin for one hour and then embedded in paraffin blocks. Sections of tissues (5 mm thickness) were treated with Diffquick stain. This treatment stains platelets in a violet to purple color.

## **2.6 Immunohistological studies**

Immunohistochemical studies were performed on the 0.5 mm thick IVC sections (control and IVC treated with 20%  $\text{FeCl}_3$  for 10 minutes) as directed in the protocol for Vectastain ABC-Alkaline phosphatase Kit (Vector Laboratories, Inc. 30 Ingold Road, Burlingame, CA, USA). An anti- GPIb $\alpha$  antibody (Emfret Analytics, Germany) was used as a primary antibody with (1:1000) dilution. The secondary antibody (biotinylated) was used with (1:200) dilution. The staining procedure stained platelets red.

## **2.7. Statistical Analysis**

Statistical analyses were done using Chi-square test from Microsoft Office Excel, this involved comparison of two groups. Values of  $p < 0.05$  are considered statistically significant.

## **2.8. Box Plots for the murine blood flow analysis**

The box plots for the murine blood flow analysis were done using SPSS (Statistical Package for Social Sciences). SPSS was used for both control and KO mice.

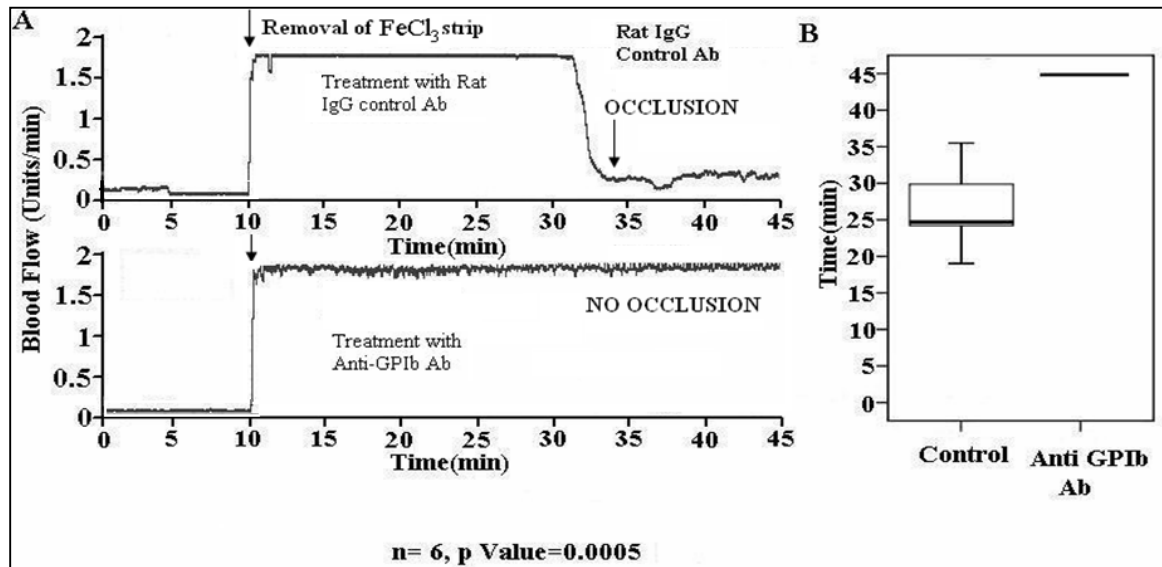
## CHAPTER 3: RESULTS

### **3.1. FeCl<sub>3</sub> treatment of the inferior vena cava causes platelet dependent stable thrombus formation.**

Stable thrombus formation can be induced in a murine carotid artery (CA) by treatment with FeCl<sub>3</sub> [24, 28, 29, 34]. FeCl<sub>3</sub>-induced stable thrombus formation in the CA is GPIb and therefore platelet dependent [2, 24, 29]. Occlusion of blood flow as a consequence of FeCl<sub>3</sub> treatment was the end point of those studies. Platelet rich thrombi were shown to be the basis of occlusion of blood flow in those studies that included characterization of the basis of occlusion [25, 28]. Although a variety of studies characterizing the role of coagulation factors and their inhibitors in FeCl<sub>3</sub>-induced thrombus formation in mouse IVC have been described [35-37], occlusion of blood flow in response to thrombus formation was not monitored. Instead, the weights of thrombi were measured.

As a prelude to investigating the role of GPIb in stable thrombus formation in the IVC, the efficacy FeCl<sub>3</sub> treatment as an inducer of stable, platelet-dependent thrombus formation in the IVC of wild type mice was characterized. In this study, the platelet requirement for thrombus formation was evaluated by monitoring blood flow following FeCl<sub>3</sub> treatment of the IVC in the presence and absence of a mixture of purified rat anti-mouse GPIb mabs [31]. Treatment of the circulating platelets with a mixture of appropriate divalent rat monoclonal anti-mouse GPIb IgG causes rapid, FcR-chain independent depletion of the platelets [31]. For our experiments, the mixture of IgG was injected into a tail vein of each anesthetized mouse after exposure of the IVC by dissection, and 15 minutes before treatment of the exposed IVC with 20% FeCl<sub>3</sub>. The

control treatment was injection of nonimmune rat IgG into the tail veins of mice subsequently treated with FeCl<sub>3</sub>. In contrast to the control treatment, treatment of the mice with the anti-GPIIb mabs resulted in depletion of at least 77% of the platelets (data not shown) by the end of blood flow monitoring period. FeCl<sub>3</sub> treatment of the IVC resulted in occlusion of blood flow in all the control animals. Five of the 7 controls treated with nonimmune IgG underwent total occlusion, 2 of the thrombi that caused total occlusion subsequently embolized prior to the end of the observation period. Two out of the 7 FeCl<sub>3</sub> treated control veins underwent stable partial occlusion; that is, total occlusion and embolus formation did not occur during the monitoring period. In contrast, the FeCl<sub>3</sub>-treated inferior vena cavae (n=7) of the mice containing the rat anti-GPIIb mabs did not occlude (Fig. 10A). Occlusion in the control animals (Fig.10A) was caused by platelet-rich thrombi (Fig.14B) consistent with the platelet requirement for thrombus formation.



**Fig.10A:** 20% FeCl<sub>3</sub>-induced IVC injury to the WT mice treated with Rat IgG (top panel) and anti-GPIb mab (bottom panel). (Blood flow was monitored for 45 minutes. The arrow represents the removal of Whatman filter paper saturated with 20% FeCl<sub>3</sub>).

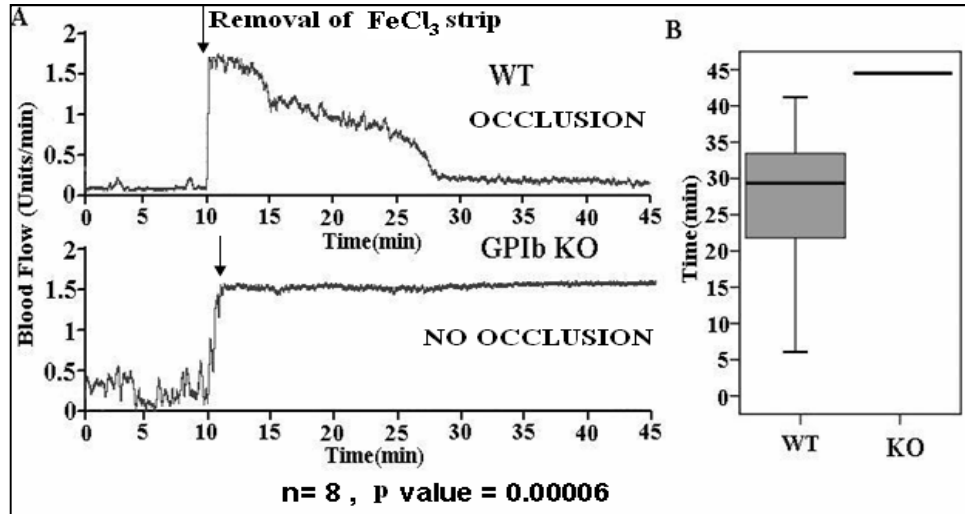
**Fig.10B:** Box plots of the time of occlusion for the control mice treated with non-immune IgG and for the mice treated with rat anti-mouse GPIb mabs. (The mean time of occlusion for the control mice was 25 minutes. The error bars depict the range of the time of occlusion for all six mice)

### 3.2. Platelet receptor GPIb $\alpha$ is required for stable thrombus formation in FeCl<sub>3</sub> induced injury in mouse inferior vena cava

The data in Fig.10 demonstrate that FeCl<sub>3</sub>-induced occlusion of the IVC is platelet dependent. Consequently, the role of the platelet specific receptor GPIb in FeCl<sub>3</sub>-induced stable thrombus formation in the IVC was investigated because the requirement for GPIb in venous (not venule) thrombosis has not been reported. In order to evaluate the requirement of GPIb in thrombus formation in IVC, GPIb KO [21] mice were included in this study. In agreement with the data reported in Fig.10A, the average time to occlusion

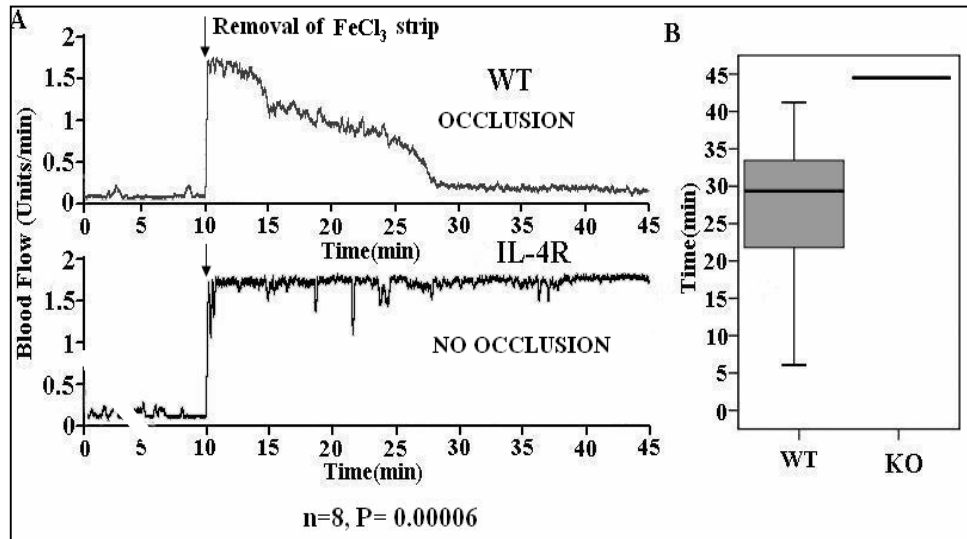


for WT mice in response to  $\text{FeCl}_3$  treatment was 27 mins. In contrast, no occlusion was observed in any of the  $\text{FeCl}_3$  (N=6) treated GPIb KO mice (Fig. 11B), ( $P=0.00006$ ). Interpretation of these results was complicated by the fact that the GPIb KO mice exhibit platelet deficiencies similar to those of Bernard-Soulier patients [21]. The GPIb KO mice have significantly lower than normal platelet count and the platelets are larger than the normal platelets (macrothrombocytopenia) [21]. So to rule out the possibility that the lack of occlusion in the GPIb KO mice was due to macrothrombocytopenia rather than the GPIb deficiency, the response of mice that do not express murine GPIb, but instead express the extracellular domain of the human interleukin-4 receptor fused with the transmembrane and cytoplasmic domains of human GPIb (IL-4R/GPIb-tg mice) was characterized [22]. Although these platelets lack the extracellular domain of GPIb, they have the normal transmembrane and cytoplasmic domains of human GPIb and therefore are normal in size and number. The IVCs of the IL-4R/GPIb-tg mice failed to occlude (Fig.12A, bottom panel) ( $P = 0.00006$ ), thereby demonstrating that the extracellular domain of GPIb is required for stable thrombus formation in response to  $\text{FeCl}_3$ -induced injury of the IVC and possibly in veins, in general.



**Fig.11A:** 20% FeCl<sub>3</sub>-induced IVC injury to the WT mice (top panel) and GPIb KO mice (bottom panel). (The arrow indicates the time of removal of the FeCl<sub>3</sub> strip. Blood flow was monitored for 45 minutes)

**Fig.11B:** Box plots of time of occlusion for control mice and GPIb KO mice. (The mean time of occlusion for WT mice was 27 minutes).



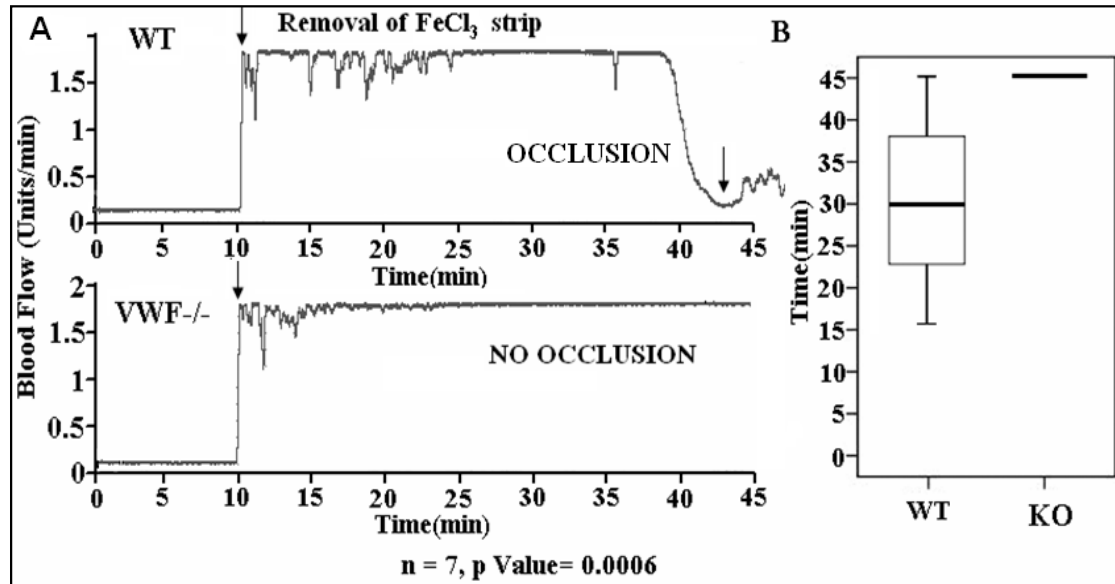
**Fig.12A:** 20% FeCl<sub>3</sub>-induced IVC injury to the WT mice (top panel) and IL-4R/GPIb $\alpha$ -tg mice (bottom panel). (Blood flow was monitored for 45 minutes. The arrow indicates the removal of FeCl<sub>3</sub> strip after 10 minutes).

**Fig.12B:** Box plots of time of occlusion for control mice and IL-4R/GPIb $\alpha$ -tg mice. (Mean time of occlusion for the WT mice was 27 minutes).

### 3.3. VWF is required for GPIb dependent thrombus formation in the mouse IVC.

The paradigm for FeCl<sub>3</sub>-induced arterial thrombosis is that the process is mediated typically but not exclusively by GPIb $\alpha$  binding to VWF on the subendothelium exposed by damage to the endothelium [8, 23]. The data presented in Figures 11B and 12B demonstrate that stable thrombus formation in the IVC resulting from FeCl<sub>3</sub> treatment is GPIb dependent. But the requirement for VWF in this process has not been reported. Accordingly, VWF deficient mice were evaluated in this system. The effect of the absence of VWF was unambiguous, none of the 7 VWF KO inferior vena cavae formed occlusive thrombi in response to FeCl<sub>3</sub>-induced injury (Fig. 13A). Seven VWF +/- (heterozygote) mice were used as controls. Out of seven, five showed occlusion and the remaining two showed a decrease in blood velocity but the injured IVCs did not

occlude completely. Lack of thrombus formation in the VWF KO mice, demonstrates that VWF is required for ferric chloride-induced thrombus formation under venous shear rates.

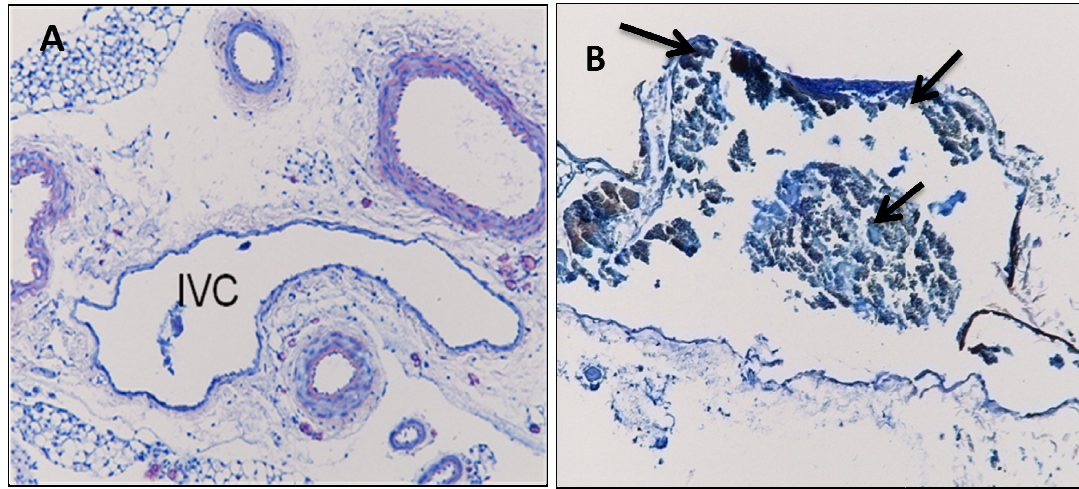


**Fig.13A:** 20% FeCl<sub>3</sub>-induced IVC injury to the WT mice (top panel) and VWF KO mice (bottom panel). (The arrow indicated the removal of FeCl<sub>3</sub> strip after the 10 minutes exposure. Blood flow was monitored for 45 minutes).

**Fig.13B:** Box plots of time of occlusion for the control mice and VWF KO mice. (The mean time of occlusion for the WT mice was 30 minutes).

### 3.4. Histological and Immunohistological studies

Thrombi formed in response to the FeCl<sub>3</sub>-induced injury to the inferior vena cavae were screened for the presence of platelets. In order to detect the presence of platelets in the occluded vessel, transverse sections of the FeCl<sub>3</sub> treated (20% FeCl<sub>3</sub> for 10 minutes) IVC was stained with Diffquick stain. Diffquick stains platelets a violet to purple color.

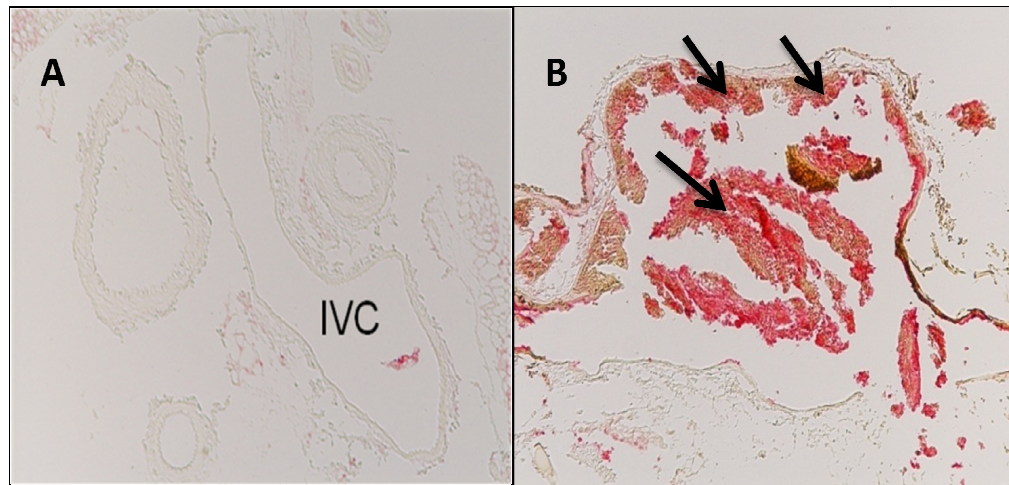


**Fig.14A:** 5  $\mu\text{m}$  thick cross section of the control IVC treated with Diffquick stain. (Magnification: 10 X)

**Fig.14B:** IVC treated with 20%  $\text{FeCl}_3$  for 10 minutes and then 0.5  $\mu\text{m}$  thick cross sections stained with Diffquick. (The arrows in Figure B indicate the violet to purple colored platelets attached to the subendothelium and present in the thrombus). (Magnification: 100 X)

Trans-sections of the non- $\text{FeCl}_3$  treated inferior vena cavae of the WT mice were used as controls. As seen in Fig. 14B, violet to purple color stained platelets can be seen attached to the subendothelium and also present in the thrombus. The presence of platelets attached to the subendothelium support the first objective of the study by demonstrating that platelets are involved in the initial step of the thrombus formation in the IVC in response to  $\text{FeCl}_3$ -induced injury.

Immunohistochemical studies were also performed on the sections of an occluded IVC previously treated with 20%  $\text{FeCl}_3$ . Alternatively, antibodies specific for platelet receptor GPIIb/IIIa were used as a marker for platelets.



**Fig.15A:** 5  $\mu$ m thick control IVC stained with the anti-GPIIb antibody.  
(Magnification: 10 X)

**Fig.15B:** 5  $\mu$ m thick cross section of IVC treated with 20%  $\text{FeCl}_3$  for 10 minutes was stained with anti-GPIIb antibody. (Arrows in Figure 14B indicate the red colored platelets in the thrombus and near the endothelium where injury was induced).  
(Magnification: 100 X)

As seen in Fig.15A, the control IVC is clear after staining with anti-GPIIb antibody but red color stained platelets can be seen attached to the subendothelium and are present in the thrombus formed in response to the  $\text{FeCl}_3$  treatment. Thus this supports the hypothesis that platelets play a role in the thrombus formation in the IVC in response to the  $\text{FeCl}_3$ -induced injury.

## CHAPTER 4: DISCUSSION

In this study, thrombus formation in the IVC of mice in response to FeCl<sub>3</sub>-induced injury was characterized. The data demonstrated that FeCl<sub>3</sub> induces thrombus formation in the IVC and that thrombus formation in the IVC is platelet, platelet receptor GPIb $\alpha$  and VWF dependent despite the presumptive low shear rate characteristic of the IVC.

Thrombus formation in high shear rate vessels such as arteries and arterioles is platelet dependent and also requires the platelet receptor GPIb $\alpha$  [28]. The initial interaction of GPIb $\alpha$  with its ligand VWF is required to form the layer of platelets that adhere to the damaged vascular wall and provide the platelet foundation for further thrombus growth [20]. The thoroughly characterized FeCl<sub>3</sub>-induced injury model was used to study thrombus formation in arteries and arterioles [7, 30].

The mechanism of arterial thrombosis is considered to be essentially different from the bases of thrombosis in veins [38]. Venous thrombus formation is thought to occur on intact endothelia as a result of stasis, coagulation and fibrinolytic abnormalities, inflammation of the endothelium and tissue factor activity [38]. Also, venous thrombi are platelet-poor; and therefore some investigators feel that platelets are not required for the thrombus formation at venous shear rates. But *in vitro* platelet adhesion experiments using perfusion chambers have demonstrated platelet adhesion to immobilized fibrinogen, fibrin and VWF at low shear rates [39]. *In vitro* studies have also shown slow thrombus growth at low shear rates [13]. But *in vivo* studies characterizing the role of platelets, GPIb $\alpha$ , and VWF at venous shear rates have not been reported and therefore this study addresses this question. Previous studies were conducted on thrombus formation in IVC in response to FeCl<sub>3</sub> induced injury, but those studies did not measure

the blood flow, instead they estimated the weight of the thrombi [33-35]. In contrast, the data described here demonstrate that thrombus formation, even at low shear rates as in IVC, in response to ferric chloride-induced injury requires platelets and GPIb $\alpha$ .

The platelet requirement for the thrombus formation in the IVC in response to FeCl<sub>3</sub> treatment was shown by using the mixture of rat anti-mouse GPIb $\alpha$  monoclonal antibodies (mab), known to deplete platelets rapidly [33]. None of the six mice treated with the mixture of mabs supported occlusion of the IVC after the FeCl<sub>3</sub> treatment. This demonstrated the requirement of platelets for thrombus formation at low shear rates. Non-immune rat IgG was used as a control for the mab studies. Each FeCl<sub>3</sub>-treated (IVC)/inferior vena cavae of the WT mice showed occlusion (n= 6, mean time of occlusion= 25 minutes).

GPIb KO mice were used to evaluate the requirement of the platelet specific receptor GPIb $\alpha$ , in thrombus formation at low shear rate. These GPIb KO mice are a model of the human- Bernard Soulier syndrome. These mice exhibit macrothrombocytopenia. The inferior vena cavae of all the eight GPIb KO mice failed to occlude in response to FeCl<sub>3</sub> treatment. Eight WT mice were used as controls and all of their (IVC)/inferior vena cavae treated with FeCl<sub>3</sub> occluded (mean time of occlusion- 27 minutes). The lack of occlusion in the GPIb KO mice shows the requirement for this platelet receptor in thrombus formation even at venous shear rates (50 s<sup>-1</sup> to 100 s<sup>-1</sup>). IL-4R/GPIb $\alpha$ -tg mice were studied to show the requirement of the extracellular domain of GPIb, and thus to rule out the possibility that the lack of thrombus formation in GPIb KO mice was due to the macrothrombocytopenia. The IL-4R/GPIb $\alpha$ -tg mice lack murine GPIb, but have the extracellular domain of the human IL-4R fused with transmembrane



and cytoplasmic domain of human- GPIb. The cytoplasmic domain of this chimeric protein ameliorates the macrothrombocytopenia, but the protein still lacks the extracellular domain of GPIb $\alpha$ . No occlusion was observed in each of the eight IL-4R/GPIb $\alpha$ -transgenic mice IVC studied. In contrast, each FeCl<sub>3</sub> treated IVC of the WT mice showed occlusion (mean time= 27 minutes). So although previous studies have shown that thrombus formation in response to ferric chloride-induced injury in venules (shear rate- 150 s<sup>-1</sup> to 200 s<sup>-1</sup>) is GPIb $\alpha$  independent [7], our data demonstrate and confirm the requirement of the extracellular domain of GPIb $\alpha$  for thrombus formation after ferric chloride treatment of the murine IVC.

VWF is the major ligand for the platelet receptor GPIb and their interaction is a requirement for thrombus formation in high shear rate vessels [3]. But previous studies carried out in high shear rate vessels such as arterioles, have found that the thrombus formation after FeCl<sub>3</sub>-induced injury does not absolutely require VWF [8]. So we studied the requirement for VWF in thrombus formation at venous shear rates. *In vitro* adhesion studies have shown that platelets can adhere to VWF at low shear rates [9]. But the *in vivo* requirement of VWF at venous shear rates in response to ferric chloride induced injury had not been reported/characterized prior to our study. We used VWF KO mice to study the thrombus formation in response to FeCl<sub>3</sub>-induced injury to the IVC. VWF +/- mice were used as controls. Out of the seven VWF KO mice, none showed occlusion of the IVC. In contrast all the WT mice occluded within 30 minutes (mean time of occlusion). Thus our data demonstrate an absolute requirement of VWF for thrombus formation in the IVC.

Pulmonary embolism is the result of unstable thrombi in veins. Characterization of thrombus formation in carotid arteries revealed stable GPIb/VWF dependent thrombus formation in response to FeCl<sub>3</sub>-induced injury. Embolization did not occur throughout the duration of observation period [30]. In contrast, thrombi in the occluded IVC embolized frequently. This difference in stability of the thrombi formed in arteries and veins may be the function of blood shear rate in veins and the physiological differences between arteries and veins. But experiments testing this concept have not been done at the physiological shear rates [40]. And so the reason for the embolization characteristic of the venous thrombosis is not understood and remains unclear.

To summarize, our studies demonstrate that FeCl<sub>3</sub>-induced thrombus formation occurs in the IVC of the mouse in a platelet, GPIb and VWF-dependent manner, despite the large diameter and the venous shear rate of the IVC. These results support the hypothesis that platelets can play an important role in DVT and VTE despite the low shear rate characteristic of veins.

## **CHAPTER 5: CONCLUSION**

Thrombus formation in the mouse inferior vena cava in response to  $\text{FeCl}_3$ -induced injury was characterized. The thrombus formation in the inferior vena cava was platelet, platelet-specific receptor GPIb and VWF dependent. This study demonstrated that stable thrombus formation can occur in a large vein with a low shear rate in a platelet, glycoprotein Ib-IX and VWF-dependent manner.

## **CHAPTER 6: FUTURE SCOPE**

The following next steps are required for this research:

1. Thrombus formation in IVC in response to 20% FeCl<sub>3</sub>-induced injury characterized in  $\alpha$ IIb $\beta$ 3 KO mice to study the importance of this receptor in thrombus growth and stability at venous shear rates as being another important and abundant receptor on the platelet membrane.
2. Thrombus formation under more physiological conditions should be characterized.

## REFERENCES

- 1 Heit JA. Venous thromboembolism: disease burden, outcomes and risk factors. *Journal of Thrombosis and Haemostasis*. 2005; **3**: 1611-7.
- 2 Andrews RK, Shen Y, Gardiner EE, Dong JF, Lopez JA, Berndt MC. The glycoprotein Ib-IX-V complex in platelet adhesion and signaling. *Thromb Haemost*. 1999; **82**: 357-64.
- 3 Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. *Current Opinion in Haematology*. 2007; **14**: 262-9.
- 4 Liu J, Pestina TI, Berndt MC, Steward SA, Jackson CW, Gartner TK. The roles of ADP and TXA in botrocetin/VWF-induced aggregation of washed platelets. *J Thromb Haemost*. 2004; **2**: 2213-22.
- 5 Wessler S. Thrombosis in Presence of Vascular Stasis. *American Journal of Medicine*. 1962; **33**: 648-66.
- 6 Wakefield TW, Strieter RM, Prince MR, Downing LJ, Greenfield LJ. Pathogenesis of venous thrombosis: a new insight. *Cardiovasc Surg*. 1997; **5**: 6-15.
- 7 Chauhan AK, Kisucka J, Lamb CB, Bergmeier W, Wagner DD. von Willebrand factor and factor VIII are independently required to form stable occlusive thrombi in injured veins. *Blood*. 2007; **109**: 2424-9.
- 8 Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest*. 2000; **106**: 385-92.
- 9 L. Badimon JJB, V.T. Turitto and V. Fuster. Role of von Willebrand in Mediating Platelet-Vessel Wall Interaction at Low Shear Rate: The Importance of Perfusion Conditions. *Blood*. 1989; **73**: 961-7.
- 10 Michelson AD. Chapter 3. *Platelets*, Second edn: American press, 2007, 45-71.
- 11 Michelson AD. Chapter 7. *Platelets*, Second edn: American press, 2007, 145-8.
- 12 Michelson AD. The Glycoprotein Ib-IX-V Complex. *Platelets*, Second edn: American press, 2007, 145-52.
- 13 Kulkarni S, Dopheide SM, Yap CL, Ravanat C, Freund M, Mangin P, Heel KA, Street A, Harper IS, Lanza F, Jackson SP. A revised model of platelet aggregation. *J Clin Invest*. 2000; **105**: 783-91.
- 14 Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, de Groot PG, Sixma JJ, Gros P. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science*. 2002; **297**: 1176-9.
- 15 Jurk K, Clemetson KJ, de Groot PG, Brodde MF, Steiner M, Savion N, Varon D, Sixma JJ, Van Aken H, Kehrel BE. Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. *FASEB J*. 2003; **17**: 1490-2.
- 16 Guerrero JA, Shafirstein G, Russell S, Varughese KI, Kanaji T, Liu J, Gartner TK, Baumler W, Jarvis GE, Ware J. In vivo relevance for platelet glycoprotein Ibalpha residue Tyr276 in thrombus formation. *J Thromb Haemost*. 2008; **6**: 684-91.
- 17 Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, McIntire LV, Berndt MC, Lopez JA. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. *J Exp Med*. 1999; **190**: 803-14.

- 18 Simon DI, Chen Z, Xu H, Li CQ, Dong J, McIntire LV, Ballantyne CM, Zhang L, Furman MI, Berndt MC, Lopez JA. Platelet glycoprotein Iba1 is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med*. 2000; **192**: 193-204.
- 19 Hoffmeister KM, Felbinger TW, Falet H, Denis CV, Bergmeier W, Mayadas TN, von Andrian UH, Wagner DD, Stossel TP, Hartwig JH. The clearance mechanism of chilled blood platelets. *Cell*. 2003; **112**: 87-97.
- 20 Lu Q, Clemetson JM, Clemetson KJ. Snake venoms and hemostasis. *J Thromb Haemost*. 2005; **3**: 1791-9.
- 21 Fernandez MF, Ginsberg MH, Ruggeri ZM, Batlle FJ, Zimmerman TS. Multimeric structure of platelet factor VIII/von Willebrand factor: the presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood*. 1982; **60**: 1132-8.
- 22 Bergmeier W, Chauhan AK, Wagner DD. Glycoprotein Iba1 and von Willebrand factor in primary platelet adhesion and thrombus formation: lessons from mutant mice. *Thromb Haemost*. 2008; **99**: 264-70.
- 23 Ware J, Russell S, Ruggeri ZM. Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. *Proc Natl Acad Sci U S A*. 2000; **97**: 2803-8.
- 24 Kanaji T, Russell S, Ware J. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. *Blood*. 2002; **100**: 2102-7.
- 25 Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, Wagner DD. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci U S A*. 1998; **95**: 9524-9.
- 26 Liu J, Fitzgerald ME, Berndt MC, Jackson CW, Gartner TK. Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIb-dependent thrombus formation in vivo. *Blood*. 2006; **108**: 2596-603.
- 27 Jirouskova M, Chereshev I, Vaananen H, Degen JL, Coller BS. Antibody blockade or mutation of the fibrinogen gamma-chain C-terminus is more effective in inhibiting murine arterial thrombus formation than complete absence of fibrinogen. *Blood*. 2004; **103**: 1995-2002.
- 28 Wang L, Miller C, Swarthout RF, Rao M, Mackman N, Taubman MB. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. *Blood*. 2009; **113**: 705-13.
- 29 Tseng MT, Dozier A, Haribabu B, Graham UM. Transendothelial migration of ferric ion in FeCl<sub>3</sub> injured murine common carotid artery. *Thromb Res*. 2006; **118**: 275-80.
- 30 Liu J, Joglekar M, Ware J, Fitzgerald ME, Lowell CA, Berndt MC, Gartner TK. Evaluation of the physiological significance of botrocetin/ von Willebrand factor in vitro signaling. *J Thromb Haemost*. 2008; **6**: 1915-22.
- 31 Konstantinides S, Ware J, Marchese P, Almus-Jacobs F, Loskutoff DJ, Ruggeri ZM. Distinct antithrombotic consequences of platelet glycoprotein Iba1 and VI deficiency in a mouse model of arterial thrombosis. *J Thromb Haemost*. 2006; **4**: 2014-21.
- 32 Marieb EN. The Cardiovascular system: blood vessels. *Human Anatomy & Physiology*, Fifth edn: Addison Wesley Longman, 2000, 718-26.

- 33 Nieswandt B, Bergmeier W, Rackebrandt K, Gessner JE, Zirngibl H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. *Blood*. 2000; **96**: 2520-7.
- 34 Bergmeier W, Piffath CL, Goerge T, Cifuni SM, Ruggeri ZM, Ware J, Wagner DD. The role of platelet adhesion receptor GPIIb/IIIa far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. *Proc Natl Acad Sci U S A*. 2006; **103**: 16900-5.
- 35 Wang X, Smith PL, Hsu MY, Gailani D, Schumacher WA, Ogletree ML, Seiffert DA. Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice. *J Thromb Haemost*. 2006; **4**: 1982-8.
- 36 Wang X, Smith PL, Hsu MY, Tamasi JA, Bird E, Schumacher WA. Deficiency in thrombin-activatable fibrinolysis inhibitor (TAFI) protected mice from ferric chloride-induced vena cava thrombosis. *J Thromb Thrombolysis*. 2007; **23**: 41-9.
- 37 Wang X, Smith PL, Hsu MY, Ogletree ML, Schumacher WA. Murine model of ferric chloride-induced vena cava thrombosis: evidence for effect of potato carboxypeptidase inhibitor. *J Thromb Haemost*. 2006; **4**: 403-10.
- 38 Mackman N. Triggers, targets and treatments for thrombosis. *Nature*. 2008; **451**: 914-8.
- 39 Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996; **84**: 289-97.
- 40 Schneider SW, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netz RR, Schneider MF. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci U S A*. 2007; **104**: 7899-903.

## APPENDIX



Dear Manali Joglekar,

Thank you for your email request. Permission is granted for you to use the material below for your thesis/dissertation subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

Best wishes,  
Laura Mothersole  
Permissions Assistant  
Wiley-Blackwell  
9600 Garsington Road  
Oxford OX4 2DQ  
UK  
Tel: +44 (0) 1865 476160  
Fax: +44 (0) 1865 471158  
Email: laura.mothersole@wiley.com

-----Original Message-----

From: PermissionsUS@wiley.com on [www.wiley.com](http://www.wiley.com) [<mailto:webmaster@wiley.com>]

Sent: Friday, October 09, 2009 1:33 PM

To: Permissions - US

Subject: Educational Photocopy Request

A01\_First\_Name: Manali  
A02\_Last\_Name: Joglekar  
A04\_Address: 3774 Walker Avenue  
A05\_City: Memphis  
A06\_State: Tennessee  
A07\_Zip: 38152  
A08\_Country: United States  
A09\_Contact\_Phone\_Number: 901-XXX-XXXX  
A10\_Fax:  
A11\_Emails: mvjoglkr@memphis.edu  
A12\_Reference:  
A27\_Book\_or\_Journal: Journal  
A13\_Book\_Title: **Journal of Thrombosis and Haemostasis**  
A14\_Book\_Author:  
A15\_Book\_ISBN:  
A15\_Book\_InStockDate:  
A16\_Journal\_Month: **November**  
A17\_Journal\_Year: **2008**  
A18\_Journal\_Volume: **6**  
A19\_Journal\_Issue\_Number: **11**  
A20\_Copy\_Pages: Page : **1916, Figure: 1**  
A22\_Maximum\_Copies: 3  
A23\_University\_Name: University of Memphis  
A24\_Course\_Name: 7996- Master's degree Thesis  
A25\_Term:  
A27\_Instructor\_Name: Dr. T. K. Gartner  
A28\_Required\_Textbook: No  
A29\_Wiley\_Author: No  
A27\_Comments\_For\_Requests: We have published this figure in our lab paper, and I would like to include this figure in my Master's degree thesis.  
A25\_Format: No  
A26\_If\_WWW\_URL:  
A28\_If\_WWW\_Password\_Access:  
A45\_WWW\_Users:  
A29\_If\_WWW\_Material\_Posted\_From:  
A30\_If\_WWW\_Material\_Posted\_To:  
:

Mothersole, Laura - Oxford [lmothersole@wiley.com] on behalf of Permission Requests  
- UK [permissionsuk@wiley.com]

Sent: Friday, October 30, 2009 8:33 AM

To: [Manali Vinay Joglekar \(mvjoglkr\)](mailto:Manali_Vinay_Joglekar_(mvjoglkr)@memphis.edu)

Dear Manali Joglekar,

Thank you for your email request. Permission is granted for you to use the material below for your thesis/dissertation subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

Best wishes,

**Laura Mothersole**

**Permissions Assistant**

**Wiley-Blackwell**

**9600 Garsington Road**

**Oxford OX4 2DQ**

**UK**

**Tel: +44 (0) 1865 476160**

**Fax: +44 (0) 1865 471158**

**Email: [laura.mothersole@wiley.com](mailto:laura.mothersole@wiley.com)**

**From:** Manali Vinay Joglekar (mvjoglkr) [mailto:mvjoglkr@memphis.edu]

**Sent:** 09 October 2009 17:41

**To:** Permission Requests - UK

**Subject:**

John Wiley & Sons, Ltd.  
Permissions Department  
The Atrium  
Southern Gate  
Chichester  
West Sussex PO 19 8SQ  
United Kingdom

Dear Sir/Madam,

This is regarding the permission to include a figure from Wiley Publication. I am a Master's Degree student in University of Memphis, TN, USA. I am writing my thesis and would like to include a figure from a paper published in Journal of Thrombosis and Haemostasis. The details of which are as below:

Journal Name: **Journal of Thrombosis and Haemostasis**

Month: **December**

Year: **2004**

Volume: **2**

Issue: **12**

Page : **2220**

Figure: **Fig 7.**

First Author: **J. Liu et al.**